Association of resistance to avian coccidiosis with single nucleotide polymorphisms in the *zyxin* gene

Y. H. Hong,* E.-S. Kim,† H. S. Lillehoj,*[1] E. P. Lillehoj,‡ and K.-D. Song§

*Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, USDA, Beltsville, MD 20705; †Department of Animal Sciences, University of Wisconsin, Madison 53706; ‡Department of Pediatrics, University of Maryland School of Medicine, Baltimore 21201; and §Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

ABSTRACT Our previous genetic studies demonstrated that resistance to avian coccidiosis is linked with microsatellite markers LEI0071 and LEI0101 on chromosome 1. In this study, the associations between parameters of resistance to coccidiosis and single nucleotide polymorphisms (SNP) in 3 candidate genes located between LEI0071 and LEI0101 [*zyxin*, *CD4*, and tumor necrosis factor receptor super family 1A (*TNFRSF1A*)] were determined. The SNP were genotyped in 24 F_1 generation and 290 F_2 generation animals. No SNP were identified in the *TNFRSF1A* gene, whereas 10 were located in the *zyxin* gene and 4 in the *CD4* gene. At various times following experimental infection of the F_2 generation with *Eimeria maxima*, BW, fecal oocyst shedding, and plasma levels of carotenoid, nitrite plus nitrate (NO_2^- + NO_3^-), and interferon-γ (IFN-γ) were measured as parameters of resistance. Single marker and haplotype-based tests were applied to determine the associations between the 14 SNP and the parameters of coccidiosis resistance. None of the *CD4* SNP were correlated with disease resistance. However, by single marker association, several of the *zyxin* SNP were significantly associated with carotenoid or NO_2^- + NO_3^- concentrations. These were the SNP at nucleotide 149 associated with carotenoid at d 3 postinfection (PI), nucleotide 187 with carotenoid at d 6 and 9 PI, and nucleotide 159 with carotenoid between d 3 and 9 PI. In addition, the *zyxin* SNP at nucleotide 191 was significantly associated with increased levels of NO_2^- + NO_3^- at d 3 PI. By haplotype association, the *zyxin* SNP also were found to be highly associated with NO_2^- + NO_3^- at d 3 PI and increased IFN-γ at d 6 PI. These results suggest that *zyxin* is a candidate gene potentially associated with increased resistance to experimental avian coccidiosis.

Key words: single nucleotide polymorphism, *zyxin*, *CD4*, disease resistance, coccidiosis

2009 Poultry Science 88:511–518
doi:10.3382/ps.2008-00344

INTRODUCTION

Avian coccidiosis is caused by ubiquitous intestinal protozoan belonging to the genera *Eimeria* that significantly impairs the growth and feed efficiency of infected chickens (Lillehoj et al., 2007). Conventional disease control methods are chemoprophylaxis with coccidiostatic drugs or vaccination with live and attenuated parasites (Dalloul and Lillehoj, 2006). Both strategies, however, suffer from serious drawbacks, and there is an increasing need to develop alternative approaches. Genetic selection of disease-resistant chickens by novel molecular genetic and functional genomics tools provides the potential to address this need, particularly when used in combination with the other established methods. With the advent of QTL mapping techniques, chicken DNA markers that are associated with growth performance and disease resistance can now be identified in particular genotypes and this information subsequently used for marker-assisted selection of breeding stocks. Single nucleotide polymorphisms, which are the most common form of DNA variation in the genome, are single base changes between individuals that can occur within or between genes. Human SNP have been reported to occur with an average frequency of about 1 in 500 to 1,000 nucleotides, and in some regions, the estimate was 1/300 (Wang et al., 1998; Dawson, 1999). The frequency of SNP in the chicken genome may be greater relative to that in the human, with a rate of 1 in 100 to 200 nucleotides (Smith et al., 2001, 2002; Vignal et al., 2002).

Identification of SNP provides a resource for building high-density genetic maps using potentially informa-

Received August 12, 2008.
Accepted November 1, 2008.
[1]Corresponding author: Hyun.Lillehoj@ars.usda.gov

Table 1. Primer sequences and PCR products used for SNP identification of the *zyxin*, *CD4*, and *TNFRSF1A* genes

Gene	Primer sequence		PCR product (bp)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
zyxin	5′-ACCCCAGGGACCCGTATGAC-3′	5′-GGGTCCTTGCGCTGCTGTG-3′	608	58	NM_001004386
CD4	5′-GGCTTCCAGGTATCATCTCT-3′	5′-ATTCTAGCTCCAGCGCATAAAAG-3′	410	50	AJ401223
TNFRSF1A	5′-CAGAGATTCAGAAGGGGTTGC-3′	5′-TAATTGCTTTTTGCTACTTCTGCT-3′	561	55	NM_001030779

tive genetic markers for a variety of applications. One such application has been the identification of genes associated with resistance/susceptibility to particular diseases (Brookes, 1999). In the case of poultry, QTL experiments were initially undertaken to identify genes associated with economically important traits such as growth rate, carcass characteristics, feed efficiency, egg production, and disease resistance (Liu et al., 2001; Lei et al., 2005; Qiu et al., 2006). Later, SNP in chicken genes encoding the vitamin D receptor, osteopontin, growth hormone, insulin, *IGF-1*, *IGFBP-2*, *PGC-1α*, *GGA1*, and *AGC1* were found to be associated with body growth, egg production, and carcass traits (Kuhnlein et al., 1997; Lei et al., 2005; Bennett et al., 2006; Qiu et al., 2006; Wu et al., 2006; Rao et al., 2007) as well as the metabolic disease tibial dyschondroplasia (Ray et al., 2006). For infectious diseases, numerous genes that control the response of chickens to *Salmonella* have been identified by SNP analysis, primarily through the pioneering studies of Sue Lamont and colleagues (Lamont et al., 2002; Liu et al., 2002; Kramer et al., 2003; Liu et al., 2003; Liu and Lamont, 2003; Malek et al., 2004; Hasenstein et al., 2006) as well as others (Hu et al., 1997; Mariani et al., 2001; Beaumont et al., 2003). Thus, there is ample evidence in the literature that SNP may be useful for identification of genes linked to resistance against poultry diseases.

In previous studies, we mapped QTL that were associated with resistance to avian coccidiosis near microsatellite marker LEI0101 on chromosome 1 (Zhu et al., 2003). Further analysis identified a second marker, LEI0071, which was closely linked to LEI0101 and more highly associated with disease resistance (Kim et al., 2006). The current study was undertaken to identify SNP in candidate genes between LEI0071 and LEI0101 that may play a role in the host response to coccidia infection and, if present, to determine whether or not these polymorphisms were associated with resistance to experimental *Eimeria maxima* infection. Three selected genes, *zyxin*, *CD4*, and tumor necrosis factor receptor super family 1A (***TNFRSF1A***), were chosen based upon their genomic location, immunological function, and previous results suggesting involvement in protective immunity to coccidiosis.

MATERIALS AND METHODS

Experimental Population

Two commercial broiler lines displaying different susceptibility to *E. maxima* were selected as resource populations to map QTL associated with resistance to coccidiosis as described previously (Zhu et al., 2003; Kim et al., 2006). Thirty male and 100 female chickens were crossed to produce F_1 chickens and 12 pairs of F_1 individuals were used to produce 290 F_2 offspring from 4 hatches. Animals were orally infected with 1.0×10^4 sporulated *E. maxima* oocysts at 4 wk of age as

Table 2. Single nucleotide polymorphisms identified in the *zyxin* and *CD4* genes

Gene	SNP	Allele 1	Allele 2	Genotype frequency[1]			MAF[2]
				1/1	1/2	2/2	
Zyxin							
	62	A	C	0.16	0.84	0.00	0.08 (A)
	82	C	T	0.92	0.08	0.00	0.04 (T)
	149	A	T	0.02	0.26	0.72	0.14 (A)
	159	A	G	0.31	0.53	0.16	0.43 (G)
	172	C	T	0.62	0.38	0.00	0.19 (T)
	187	C	T	0.73	0.24	0.03	0.15 (T)
	191	A	G	0.00	0.17	0.83	0.08 (A)
	378	A	C	0.00	0.38	0.62	0.19 (A)
	Intron 1	A	G	0.22	0.41	0.37	0.43 (A)
	Intron 2	A	T	0.23	0.41	0.36	0.43 (A)
CD4							
	11591	C	T	0.15	0.48	0.37	0.39 (C)
	11697	C	T	0.64	0.34	0.02	0.19 (T)
	11719	A	G	0.64	0.34	0.02	0.19 (G)
	11790	A	T	0.16	0.48	0.37	0.40 (A)

[1]1 = allele 1; 2 = allele 2.

[2]MAF = minor allele frequency.

described (Zhu et al., 2003). Body weights, fecal oocyst shedding, and plasma concentrations of carotenoid, $NO_2^- + NO_3^-$, and interferon-γ (**IFN-γ**) were measured on d 0, 3, 6, and 9 postinfection (**PI**) as described previously (Zhu et al., 2003). One fecal sample per bird was collected during the time period from d 5 to 9 PI.

Genomic DNA Isolation, PCR Amplification and Sequencing, and Genomic Sequencing

Genomic DNA was prepared from erythrocytes using the GenElute Blood Genomic DNA kit according to the manufacturer's protocol (Sigma, St. Louis, MO). The PCR primer sequences specific for the *zyxin*, *CD4*, and *TNFRSF1A* genes are shown in Table 1. The PCR were performed using ABgene Thermo-Start PCR Master mix (Thermo Fisher Scientific, Rockford, IL). Twenty-five microliters of the PCR reaction contained 50 ng of chicken genomic DNA, 10 pmol of each primer, 0.2 m*M* of each dNTP, 0.625 U of DNA polymerase, 1× reaction buffer, and 1.5 m*M* $MgCl_2$. The PCR products were precipitated in ethanol, dissolved in H_2O, and sequenced using internal primers with an ABI 3730 DNA analyzer (Applied Biosystem, Foster City, CA). The SNP genotyping was performed by genomic DNA sequencing with the same gene-specific internal primers. The SNP genotypes of each gene were identified by alignment of sequences using BLAST software (bl2seq; Tatusova and Madden, 1999) and analyzed with the BioEdit biological sequence alignment editor (Hall, 1999).

Association Testing

Associations between SNP genotypes or haplotypes and disease phenotypes were evaluated using the following linear models 1 and 2:

$$\text{Model 1 (SNP genotypes): } y = \mu + F + H + S + Wg + e;$$

$$\text{Model 2 (SNP haplotypes): } y = \mu + F + H + S + Xb + e,$$

where y is the phenotype, μ is the overall phenotypic mean, F is the family effect, H is the hatch effect, S is the sex effect, W is an incidence matrix of genotype, g and b are vectors of the regression coefficient, X is an incidence matrix of haplotype, and e is the residual error. The incidence matrix X indicated the haplotype copy number (0, 1, or 2) in the individual. The size of vector b was variable for the haplotype-based model (size of $b = [N - 1] \times 1$) where N is the major haplotype (>1%). In the model of haplotype analysis, the allele substitution effect was estimated. The effect of the most frequent haplotype of phenotype was set to zero and haplotype substitution effect (b) of the other haplotype was estimated (Khatib et al., 2005). Family effect (F) and hatch effect (H) are added as random effects; and sex effect (S), genotype, and haplotype effect are included as fixed effects. The distribution of BW and plasma carotenoid concentrations satisfied assumption of normality. The number of fecal oocyst and plasma concentrations of IFN-γ and $NO_2^- + NO_3^-$ were used after log-transformations. Statistical analysis was performed using the lme4 package in the R Project for Statistical Computing (www.r-project.org).

Linkage Disequilibrium Measurement

Linkage disequilibrium (**LD**) between all pairs of SNP was analyzed using the genetics package of the R Project. Pairwise D' (Lewontin, 1964) and squared correlation coefficient r^2 (Hill and Robertson, 1968) between SNP were determined to measure the strength of LD.

RESULTS

SNP Genotyping and LD

Sequence analysis of PCR-amplified genomic DNA from a reference population of 314 chickens (24 F_1 and 290 F_2 generation) was used to identify SNP in the *zyxin, CD4*, and *TNFRSF1A* genes. No SNP were found in the *TNFRSF1A* gene, whereas 10 SNP were identified in *zyxin* and 4 in *CD4* (Table 2). The 608-bp PCR product of the *zyxin* gene (GenBank accession No. NM_001004386) contained of 5 nonsynonymous (i.e., resulting in an amino acid change) SNP (nucleotides 62, 82, 149, 172, and 187) and 3 synonymous (not resulting in an amino acid change) SNP (nucleotides 159, 191, and 378), all within exons, and 2 intronic SNP (Introns 1 and 2). The 410-bp PCR product of the *CD4* gene (GenBank accession No. AJ401223) included 4 nonsynonymous SNP (nucleotides 11591, 11697, 11719, and 11790) all within the largest exon. The minor allele frequencies of 3 SNP in the *zyxin* gene (nucleotides 62, 82, and 191) were less than 0.1 (Table 2).

Across all SNP in both genes, the range of LD (D') varied between 0.63 and 1.00 within *zyxin* gene (Table 3, upper right section). The strength of LD within the *CD4* gene ($D' > 0.99$, $r^2 = 0.17$ ~1.00) was greater compared with the corresponding LD values between the 2 genes ($D' = 0.23$ ~1.00, $r^2 < 0.10$). Squares of correlation coefficients (r^2) among all SNP varied between 0.01 and 0.75.

Association of zyxin and CD4 SNP with Parameters of Resistance to Coccidiosis

Body weights, fecal oocyst shedding, and plasma levels of carotenoid, nitrite plus nitrate ($NO_2^- + NO_3^-$), and IFN-γ were measured at d 0, 3, 6, or 9 PI (or a

Table 3. Linkage disequilibrium in *zyxin* and *CD4* SNP[1]

	Zyxin										*CD4*			
Item	62	82	149	159	172	187	191	378	Int1	Int2	11591	11697	11719	11790
Zyxin														
62		0.96	0.63	1.00	0.99	0.99	0.93	1.00	1.00	1.00	0.23	0.25	0.25	0.23
82	0.00		0.98	1.00	1.00	0.98	0.93	1.00	0.99	0.99	0.99	1.00	1.00	0.99
149	0.01	0.00		1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	0.40	0.40	1.00
159	0.16	0.04	0.11		1.00	1.00	1.00	1.00	1.00	1.00	0.46	0.15	0.15	0.46
172	0.01	0.22	0.02	0.18		0.99	0.99	1.00	1.00	1.00	0.20	1.00	1.00	0.20
187	0.01	0.00	0.75	0.08	0.01		0.99	1.00	1.00	1.00	1.00	0.47	0.47	1.00
191	0.08	0.00	0.01	0.08	0.00	0.01		0.99	1.00	1.00	1.00	1.00	1.00	1.00
378	0.02	0.10	0.04	0.41	0.44	0.03	0.01		1.00	1.00	0.27	1.00	1.00	0.27
Int1	0.05	0.01	0.10	0.32	0.06	0.08	0.03	0.13		1.00	0.29	0.29	0.29	0.29
Int2	0.05	0.01	0.11	0.33	0.06	0.08	0.03	0.14	0.05		0.34	0.31	0.31	0.34
CD4														
11591	0.01	0.01	0.10	0.07	0.00	0.08	0.09	0.03	0.00	0.10		1.00	1.00	1.00
11697	0.02	0.07	0.10	0.00	0.03	0.10	0.01	0.07	0.00	0.02	0.17		1.00	1.00
11719	0.02	0.07	0.10	0.00	0.03	0.10	0.01	0.07	0.00	0.02	0.17	1.00		1.00
11790	0.01	0.01	0.10	0.07	0.00	0.08	0.09	0.03	0.00	0.10	1.00	0.17	0.17	

[1]*D′* values are listed in the upper right section, and r^2 values are listed in the lower left section. Int1 = intron 1; Int2 = intron 2.

combination of these) as parameters of resistance to experimental *E. maxima* coccidiosis. A total of 224 comparisons were made [(16 traits × 10 *zyxin* SNP) + (16 traits × 4 *CD4* SNP)]. Table 4 lists the 16 individual disease traits that were measured and the individual *zyxin* and *CD4* SNP that was most highly associated with each trait. Of these single marker associations, 5 *zyxin* SNP were observed to be statistically significant ($P < 0.05$): (1) allele T of SNP 149 with increased carotenoid at d 3 PI ($P = 0.008$), (2) allele T of SNP 187 with increased carotenoid at d 6 PI ($P = 0.011$), (3) allele G of SNP 187 with increased carotenoid at d 9 PI ($P = 0.007$), (4) allele A of SNP 159 with a greater increase in carotenoid between d 3 and 9 PI ($P = 0.007$), and (5) SNP 191 with increased $NO_2^- + NO_3^-$ at d 3 PI ($P = 0.006$). In addition, the *CD4* SNP 11697 was significantly associated with a greater increase in carotenoid between d 3 and 9 PI ($P = 0.049$).

The allele substitution effects of the *zyxin* and *CD4* SNP on the levels of plasma carotenoid at d 3, 6, 9, and 3 to 9 PI as well as the levels of $NO_2^- + NO_3^-$ at d 3 PI are shown in Table 5. Several statistically significant effects of substitution from allele 1 to allele 2 were noted in the *zyxin* gene, such as at SNP 149, 172, and 187 with carotenoid levels on d 3, 6, and 9 PI, and SNP 191 with $NO_2^- + NO_3^-$ levels at d 3 PI. In the case of the *CD4* gene, SNP 11697 and 11719 were significantly associated with plasma carotenoid at d 3–9 PI.

Table 4. Associations between *zyxin* and *CD4* SNP and disease-resistant traits

	zyxin		*CD4*	
Trait[1]	SNP	*P*-value[2]	SNP	*P*-value[2]
bw0	62	0.353	11591	0.654
bw3	62	0.572	11591	0.597
bw6	Intron1	0.586	11697	0.506
bw9	172	0.590	11591	0.494
bw6–9	191	0.081	11719	0.266
crnt3	149	0.008	11697	0.328
crnt6	187	0.011	11719	0.156
crnt9	187	0.007	11697	0.143
crnt3–9	159	0.007	11697	0.049
oocyst9	187	0.087	11591	0.541
NO3	191	0.006	11697	0.639
NO6	82	0.110	11591	0.202
NO9	187	0.436	11697	0.209
IFN3	191	0.078	11719	0.416
IFN6	62	0.224	11719	0.232
IFN9	191	0.084	11719	0.171

[1]bw0, bw3, bw6, and bw9 = BW on d 0, 3, 6, and 9 postinfection (PI); bw6–9 = BW gain between d 6 and 9 PI; crnt3, cnrt6, and cnrt9 = plasma carotenoid concentrations on 3, 6, and 9 PI; crnt3–9 = increase in plasma carotenoid between d 3 and 9 PI; oocyst9 = the number of fecal oocysts at d 9 PI; NO3, NO6, and NO9 = plasma $NO_2^- + NO_3^-$ concentrations on d 3, 6, and 9 PI; IFN3, IFN6, and IFN9 = plasma IFN-γ concentrations on d 3, 6, and 9 d PI; oocyst = the number of shedding oocysts.

[2]The most significant association marker-trait associations.

Table 5. Single marker substitution effects with selected disease resistance traits[1]

Gene	SNP	crnt3	crnt6	crnt9	crnt3–9	NO3
Zyxin						
	62	0.151	0.035	0.032	0.185	0.112
	82	0.174	0.095	0.103	0.050	0.004
	149	-0.234[2]	-0.200[3]	−0.147	0.030	0.025
	159	0.001	−0.097	0.001	0.136[2]	0.006
	172	−0.046	-0.189[3]	−0.001	0.150[3]	0.026
	187	0.180	0.270[2]	0.253[3]	−0.085	−0.040
	191	0.007	0.045	−0.016	−0.006	-0.119[2]
	378	−0.044	-0.202[3]	−0.004	0.159[3]	0.023
	Intron 1	0.0860	−0.004	0.054	0.081[3]	−0.010
	Intron 2	0.0860	−0.003	0.052	0.080[3]	−0.010
CD4						
	11591	0.009	0.043	−0.019	−0.011	−0.009
	11697	0.076	−0.097	0.108	0.133[3]	−0.020
	11719	0.042	−0.124	0.059	0.132[3]	0.011
	11790	0.009	0.043	−0.019	−0.011	−0.009

[1]crnt3, cnrt6, and cnrt9 = plasma carotenoid concentrations on 3, 6, and 9 postinfection (PI); crnt3–9 = increase in plasma carotenoid between d 3 and 9 PI; NO3 = plasma NO_2^- + NO_3^- concentration on d 3.

[2]Denotes $P < 0.01$.

[3]Denotes $P < 0.05$.

Haplotype Classification Analysis of zyxin and CD4

A total of 10 *zyxin* SNP haplotypes were found in the 12 families, of which the 7 most frequent (arbitrarily designated A, B, C, D, E, F, and G) accounted for 97.5% of the total observed (Table 6). Haplotypes A-F were discriminated from the most common haplotype G by multiple base changes, recombination, or both (Table 6). A total of 5 *CD4* SNP haplotypes were observed, of which the 3 most frequent (arbitrarily designated I, II, III) accounted for 98.7% of the total.

Association of zyxin and CD4 SNP Haplotypes with Parameters of Resistance to Coccidiosis

The haplotype association test was used to determine the relationship between the 16 parameters of coccidiosis resistance determined above and the haplotypes of the *zyxin* and *CD4* gene SNP. Haplotypes of both genes were included in the analysis if their frequency was greater than 0.01. As shown in Table 7, the *zyxin* was associated with the 3 most common parameters of coccidiosis disease resistance, carotenoid level at d 9 PI (crnt9), NO_2^- + NO_3^- at d 3 PI (NO3), and serum IFN-γ at d 6 PI (IFN6). In contrast, the *CD4* was not significantly associated with any of the disease resistance traits.

The allele substitution effect of *zyxin* haplotype G versus A-F and of *CD4* haplotype III versus I and II was determined for these 3 traits. Compared with the most frequent haplotype G, haplotypes C and D were significantly associated with carotenoid levels at d 9 PI, whereas haplotype D was associated with increased NO_2^- + NO_3^- levels at d 3 PI (Table 8). Haplotype D was significantly associated with carotenoid and NO_2^- + NO_3^- in the same direction. Finally, the substitution effect of haplotype B was significantly associated with IFN-γ levels at d 6 PI. Associations between all of the traits evaluated and *CD4* gene haplotypes were not detected.

Table 6. Haplotype of the *zyxin* and *CD4* gene SNP

Zyxin			*CD4*		
Haplotype	62–82–149–159–172–187–191–378-I1-I2[1]	Frequency	Haplotype	11591–11697–11719–11790[2]	Frequency
A	A-C-T-G-C-C-G-C-G-T	0.079	I	C-C-A-A	0.377
B	C-C-A-A-C-T-G-C-G-T	0.145	II	T-T-G-T	0.181
C	C-C-T-G-C-C-A-C-G-T	0.074	III	T-C-A-T	0.429
D	C-C-T-G-C-C-G-C-G-T	0.039			
E	C-C-T-G-T-C-G-A-G-T	0.179			
F	C-T-T-G-C-C-G-C-G-T	0.036			
G	C-C-T-A-C-C-G-C-A-A	0.423			
All other haplotypes		0.025	All other haplotypes		0.013

[1]Alleles of the 10 *zyxin* SNP; I1 = Intron 1 SNP; I2 = Intron 2 SNP.

[2]Alleles of the 4 *CD4* SNP.

Table 7. Associations of *zyxin* and *CD4* gene haplotypes with disease resistance traits

	P-value	
Trait[1]	*zyxin*	*CD4*
bw0	0.94	0.82
bw3	0.98	0.71
bw6	0.96	0.56
bw9	0.94	0.53
bw6–9	0.58	0.72
crnt3	0.12	0.83
crnt6	0.34	0.36
crnt9	0.10	0.62
crnt3–9	0.30	0.41
oocyst	0.34	0.60
NO3	0.02	0.80
NO6	0.65	0.50
NO9	0.96	0.53
IFN3	0.36	0.61
IFN6	0.04	0.46
IFN9	0.46	0.50

[1]bw0, bw3, bw6, and bw9 = BW on d 0, 3, 6, and 9 postinfection (PI); bw6–9 = BW gain between d 6 and 9 PI; crnt3, cnrt6, and cnrt9 = plasma carotenoid concentrations on 3, 6, and 9 PI; crnt3–9 = increase in plasma carotenoid between d 3 and 9 PI; oocyst = the number of shedding oocysts; NO3, NO6, and NO9 = plasma NO_2^- + NO_3^- concentrations on d 3, 6, and 9 PI; IFN3, IFN6, and IFN9 = plasma IFN-γ concentrations on d 3, 6, and 9 d PI.

DISCUSSION

The foundation for this study originated from our prior reports that identified QTL that were linked to the LEI0071 and LEI0101 genetic markers, located at 79.46 and 84.53 Mb, respectively, on chromosome 1, and that were associated with resistance to experimental avian coccidiosis (Zhu et al., 2003; Kim et al., 2006). Although these 2 markers supplied important information on the genetics of resistance to *Eimeria* infection, the region in which they are located is sufficiently large (>5.0 Mb) and may contain hundreds, if not thousands, of candidate genes that are responsible for the observed phenotype. As an initial approach to identify genetic elements that may influence the host response to coccidia infection, we selected 3 genes for further analysis, *zyxin* located at 80.67 Mb, *CD4* at 80.36 Mb, and *TNFRSF1A* at 82.68 Mb. Our results showed that a statistically significant association of resistance to avian coccidiosis occurred with SNP in the *zyxin* gene.

We selected the candidate genes based upon their proximity to the previously identified QTL, their known roles in protective immunity, and our previous microarray studies that identified genes whose expression was upregulated following *Eimeria* infection. For example, the *zyxin* gene transcript was increased >2.0-fold at 5 to 6 d PI with coccidia parasites compared with uninfected animals using an avian intestinal intraepithelial lymphocyte cDNA microarray (Kim et al., 2008). *Zyxin* encodes a protein that functions as a component of focal adhesion complexes, regulates actin filament assembly, and promotes attachment of epithelial cells to the extracellular matrix (Zaidel-Bar et al., 2003). Thus, it is tempting to speculate that one of the host responses to *Eimeria* infection involves upregulation of the expression of genes such as *zyxin* that mediate focal adhesion, thereby promoting intestinal epithelial barrier formation against parasite invasion.

The 2 other genes chosen for this study, *CD4* and *TNFRSF1A*, are well-characterized components in host immunity to infectious microorganisms. The *CD4* gene product is a T cell surface glycoprotein that serves in conjunction with the T cell receptor as a co-receptor of the T cell receptor complex and that binds in an antigen-independent manner to major histocompatibility complex class II molecules on antigen presenting cells (Miceli and Parnes, 1993). Thus, *CD4* is intimately involved in initiation of immune responses. However, of the 4 *CD4* gene SNP and their associated 3 major haplotypes identified in the 12 chicken families, none were significantly associated with coccidiosis resistance traits. It is important to point out that the *CD4* SNP markers are located within a relatively small genomic region (>200 bp), which may limit the ability to identify association with disease resistance traits. Considering the importance of *CD4* in the adaptive immune response and the close proximity of the *CD4* gene to the QTL previously identified, further studies are needed to determine if other *CD4* genetic markers may be associated with resistance to avian coccidiosis. The *TNFRSF1A* (formerly termed *TNFR1*) is the gene encoding the 55 kDa *TNF-*α receptor. Through binding

Table 8. *Zyxin* haplotype substitution effect on selected traits

	Trait[1]					
	crnt9		NO3		IFN6	
Haplotype	Effect (SD)	*P*-value	Effect (SD)	*P*-value	Effect (SD)	*P*-value
A	0.09 (0.31)	0.121	0.15 (0.21)	0.488	−0.03 (0.21)	0.488
B	0.56 (0.27)	0.821	−0.01 (0.18)	0.929	0.55 (0.43)	0.002
C	0.01 (0.31)	0.011	−0.24 (0.21)	0.808	0.01 (0.45)	0.983
D	−0.19 (0.39)	0.033	−0.32 (0.26)	0.001	−0.19 (0.53)	0.492
E	−0.23 (0.27)	0.692	0.03 (0.18)	0.834	−0.24 (0.39)	0.121
F	−0.02 (0.38)	0.286	−0.15 (0.25)	0.980	−0.02 (0.57)	0.949

[1]crnt9 = plasma carotenoid concentration on 9 d postinfection; NO3 = plasma NO_2^- + NO_3^- concentration on d 3; IFN6 = plasma IFN-γ concentration on d 6.

to *TNFRSF1A*, *TNF-α* functions as a key mediator in the inflammatory response with pleiotropic activities, including increased expression of adhesion molecules, induction of cytokine secretion, activation of leukocytes, and host defense against intracellular pathogens (Rezaei, 2006). Although our experiments were unable to detect SNP in the chicken *TNFRSF1A* gene, they do not preclude the potential role that this gene and its encoded protein may play during the host inflammatory response to coccidiosis.

Historically, increased BW gain and decreased oocyst fecal shedding generally have been considered as the most meaningful parameters that are associated with increased resistance to experimental *Eimeria* infection, for example, following vaccination with attenuated parasites compared with nonvaccinated controls. In addition, other phenotypic markers also have been shown to positively correlate with disease resistance. Particularly, plasma carotenoid concentrations were previously shown to be a more sensitive measurement of avian coccidiosis than BW gain (Conway et al., 1990, 1993). In support of this observation, in this study, 10 *zyxin* gene single markers that were significantly associated with plasma carotenoid concentrations were detected in the present study. Whether *zyxin* has a direct or indirect effect on plasma carotenoid concentration is currently unclear. Interestingly, *zyxin* gene expression is upregulated in the nucleus by thymosin β4 and, similar to carotenoid, is implicated along with thymosin β4 in cell migration, angiogenesis, wound healing, and tumor metastasis (Moon et al., 2006).

Although the increase in carotenoid between d 3 to 9 PI was significantly correlated with oocyst shedding ($r^2 = 0.61$), only weak evidence of association between the presence of the *zyxin* markers and oocyst shedding was detected. Our previous studies examining segregation of the LEI0071 and LEI0101 QTL in relation to oocyst shedding was performed using 4 chicken families (Zhu et al., 2003; Kim et al., 2006). These families were included in the current analysis. Although haplotype association testing detected an association between *zyxin* gene haplotypes and oocyst shedding ($P = 0.001$) in these 4 families, 2 SNP associations (SNP intron 1 and intron 2) with oocyst shedding were supported at the level of $P < 0.1$. Furthermore, when the analysis was expanded to the 12 families in the current study, the association between *zyxin* haplotypes and oocyst shedding was lost.

The SNP used in this report were selected based on LD and allele frequency. Single marker association analysis was used considering low LD estimated by the square of correlation coefficient (r^2) between markers. In addition to single marker associations, haplotype association testing was also applied based on the number of major haplotype in the genes and LD measured by D'. Both types of analyses for the *zyxin* gene detected significant associations with $NO_2^- + NO_3^-$ levels at d 3 PI. Haplotype-based testing also detected a significant association between the less frequent *zyxin* B haplotype and increased IFN-γ at d 6 PI, which was not identified by single marker association analysis. Thus, these 2 types of association analyses provided complementary tools for identification of potential marker-trait correlations. It has been noted that if a causal connection between genetic markers and a given phenotype is truly driven by a single SNP, then the haplotype-based approach may perform worse than the one-SNP-at-a-time approach (Clark, 2004).

In conclusion, single marker association analysis for 16 traits of coccidiosis resistance showed that 5 *zyxin* SNP and 1 *CD4* SNP were significantly associated with some of the traits. Additional markers of resistance were noted by haplotype association analysis. Future studies will be needed to identify additional markers in other chicken families that may be associated with coccidiosis resistance phenotypes in other broiler chicken families and to develop markers for other candidate genes relevant to protective immunity. Ultimately, genetic breeding programs to select for these and other markers may provide chicken lines with enhanced resistance to avian coccidiosis.

ACKNOWLEDGMENTS

This project was supported, in part, by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (NRI grant #2004–35204–14798).

REFERENCES

Beaumont, C., J. Protais, F. Pitel, G. Leveque, D. Malo, F. Lantier, F. Plisson-Petit, P. Colin, M. Protais, P. Le Roy, J. M. Elsen, D. Milan, I. Lantier, A. Neau, G. Salvat, and A. Vignal. 2003. Effect of two candidate genes on the *Salmonella* carrier state in fowl. Poult. Sci. 82:721–726.

Bennett, A. K., P. Y. Hester, and D. E. Spurlock. 2006. Polymorphisms in vitamin D receptor, osteopontin, insulin-like growth factor 1 and insulin, and their associations with bone, egg and growth traits in a layer–broiler cross in chickens. Anim. Genet. 37:283–286.

Brookes, A. J. 1999. The essence of SNPs. Gene 234:177–186.

Clark, A. G. 2004. The role of haplotypes in candidate gene studies. Genet. Epidemiol. 27:321–333.

Conway, D. P., M. E. McKenzie, and A. D. Dayton. 1990. Relationship of coccidial lesion scores and weight gain in infections of *Eimeria acevulina, E. maxima* and *E. tenella* in broilers. Avian Pathol. 19:489–496.

Conway, D. P., K. Sasai, S. M. Gaafar, and C. D. Smothers. 1993. Effects of different levels of oocyst inocula of *Eimeria acervulina, E. tenella*, and *E. maxima* on plasma constituents, packed cell volume, lesion scores, and performance in chickens. Avian Dis. 37:118–123.

Dalloul, R. A., and H. S. Lillehoj. 2006. Poultry coccidiosis: Recent advancements in control measures and vaccine development. Expert Rev. Vaccines 5:143–163.

Dawson, E. 1999. SNP maps: More markers needed? Mol. Med. Today 5:419–420.

Hall, T. A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98.

Hasenstein, J. R., G. Zhang, and S. J. Lamont. 2006. Analyses of five gallinacin genes and the *Salmonella enterica* serovar Enteritidis response in poultry. Infect. Immun. 74:3375–3380.

Hill, W. G., and A. Robertson. 1968. Linkage disequilibrium in finite populations. Theor. Appl. Genet. 38:226–231.

Hu, J., N. Bumstead, P. Barrow, G. Sebastiani, L. Olien, K. Morgan, and D. Malo. 1997. Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. Genome Res. 7:693–704.

Khatib, H., E. Heifetz, and J. C. M. Dekkers. 2005. Association of the protease inhibitor gene with production traits in Holstein dairy cattle. J. Dairy Sci. 88:1208–1231.

Kim, C. H., H. S. Lillehoj, T. W. Bliss, C. L. Keeler Jr., Y. H. Hong, D. W. Park, M. Yamage, W. Min, and E. P. Lillehoj. 2008. Construction and application of an avian intestinal intraepithelial lymphocyte cDNA microarray (AVIELA) for gene expression profiling during Eimeria maxima infection. Vet. Immunol. Immunopathol. 124:341–354.

Kim, E. S., Y. H. Hong, W. Min, and H. S. Lillehoj. 2006. Fine-mapping of coccidia-resistant quantitative trait loci in chickens. Poult. Sci. 85:2028–2030.

Kramer, J., M. Malek, and S. J. Lamont. 2003. Association of twelve candidate gene polymorphisms and response to challenge with *Salmonella* Enteritidis in poultry. Anim. Genet. 34:339–348.

Kuhnlein, U., L. Ni, S. Weigend, J. S. Gavora, W. Fairfull, and D. Zadworny. 1997. DNA polymorphisms in the chicken growth hormone gene: Response to selection for disease resistance and association with egg production. Anim. Genet. 28:116–123.

Lamont, S. J., M. G. Kaiser, and W. Liu. 2002. Candidate genes for resistance to *Salmonella* Enteritidis colonization in chickens as detected in a novel genetic cross. Vet. Immunol. Immunopathol. 87:423–428.

Lei, M. M., Q. H. Nie, X. Peng, D. X. Zhang, and X. Q. Zhang. 2005. Single nucleotide polymorphisms of the chicken insulin-like factor binding protein 2 gene associated with chicken growth and carcass traits. Poult. Sci. 84:1191–1198.

Lewontin, R. C. 1964. The interaction of selection and linkage II. Optimal model. Genetics 50:757–782.

Lillehoj, H. S., C. H. Kim, C. L. Keeler Jr., and S. Zhang. 2007. Immunogenomic approaches to study host immunity to enteric pathogens. Poult. Sci. 86:1491–1500.

Liu, H. C., H. J. Kung, J. E. Fulton, R. W. Morgan, and H. H. Cheng. 2001. Growth hormone interacts with the Marek's disease virus SORF2 protein and is associated with disease resistance in chicken. Proc. Natl. Acad. Sci. USA 98:9203–9208.

Liu, W., M. G. Kaiser, and S. J. Lamont. 2003. Natural resistance-associated macrophage protein 1 gene polymorphisms and response to vaccine against or challenge with *Salmonella* Enteritidis in young chicks. Poult. Sci. 82:259–266.

Liu, W., and S. J. Lamont. 2003. Candidate gene approach: Potentional association of caspase-1, inhibitor of apoptosis protein-1, and prosaposin gene polymorphisms with response to *Salmonella* Enteritidis challenge or vaccination in young chicks. Anim. Biotechnol. 14:61–76.

Liu, W., M. M. Miller, and S. J. Lamont. 2002. Association of MHC class I and class II gene polymorphisms with vaccine or challenge response to S*almonella* Enteritidis in young chicks. Immunogenetics 54:582–590.

Malek, M., J. R. Hasenstein, and S. J. Lamont. 2004. Analysis of chicken TLR4, CD28, MIF, MD-2, and LITAF genes in a S*almonella* Enteritidis resource population. Poult. Sci. 83:544–549.

Mariani, P., P. A. Barrow, H. H. Cheng, M. M. Groenen, R. Negrini, and N. Bumstead. 2001. Localization to chicken chromosome 5 of a novel locus determining salmonellosis resistance. Immunogenetics 53:786–791.

Miceli, M. C., and J. R. Parnes. 1993. Role of CD4 and CD8 in T cell activation and differentiation. Adv. Immunol. 53:59–122.

Moon, H. S., S. Even-Ram, H. K. Kleinman, and H. J. Cha. 2006. Zyxin is upregulated in the nucleus by thymosin β4 in SiHa cells. Exp. Cell Res. 312:3425–3431.

Qiu, F. F., Q. H. Nie, C. L. Luo, D. X. Zhang, S. M. Lin, and X. Q. Zhang. 2006. Association of single nucleotide polymorphisms of the insulin gene with chicken early growth and fat deposition. Poult. Sci. 85:980–985.

Rao, Y., X. Shen, M. Xia, C. Luo, Q. Nie, D. Zhang, and X. Zhang. 2007. SNP mapping of QTL affecting growth and fatness on chicken GGA1. Genet. Sel. Evol. 39:569–582.

Ray, S. A., P. B. Drummond, L. Shi, G. R. McDaniel, and E. J. Smith. 2006. Mutation analysis of the aggrecan gene in chickens with tibial dyschondroplasia. Poult. Sci. 85:1169–1172.

Rezaei, N. 2006. TNF-receptor-associated periodic syndrome (TRAPS): An autosomal dominant multisystem disorder. Clin. Rheumatol. 25:773–777.

Smith, E. J., L. Shi, P. Drummond, L. Rodriguez, R. Hamilton, S. Ramlal, G. Smith, K. Pierce, and J. Foster. 2001. Expressed sequence tags for the chicken genome from a normalized 10-day-old White Leghorn whole embryo cDNA library: 1. DNA sequence characterization and linkage analysis. J. Hered. 92:1–8.

Smith, E. J., L. Shi, and G. Smith. 2002. Expressed sequence tags for the chicken genome from a normalized 10-day-old White Leghorn whole-embryo cDNA library. 3. DNA sequence analysis of genetic variation in commercial chicken populations. Genome 45:261–267.

Tatusova, T. A., and T. L. Madden. 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. 174:247–250.

Vignal, A., D. Milan, M. SanCristobal, and A. Eggen. 2002. A review on SNP and other types of molecular markers and their use in animal genetics. Genet. Sel. Evol. 34:275–305.

Wang, D. G., J. B. Fan, C. J. Siao, A. Berno, P. Young, R. Sapolsky, G. Ghandour, N. Perkins, E. Winchester, J. Spencer, L. Kruglyak, L. Stein, L. Hsie, T. Topaloglou, E. Hubbell, E. Robinson, M. Mittmann, M. S. Morris, N. Shen, D. Kilburn, J. Rioux, C. Nusbaum, S. Rozen, T. J. Hudson, R. Lipshutz, M. Chee, and E. S. Lander. 1998. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 280:1077–1082.

Wu, G. Q., X. M. Deng, J. Y. Li, N. Li, and N. Yang. 2006. A potential molecular marker for selection against abdominal fatness in chickens. Poult. Sci. 85:1896–1899.

Zaidel-Bar, R., C. Ballestrem, Z. Kam, and B. Geiger. 2003. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J. Cell Sci. 116:4605–4613.

Zhu, J. J., H. S. Lillehoj, P. C. Allen, C. P. Van Tassell, T. S. Sonstegard, H. H. Cheng, D. Pollock, M. Sadjadi, W. Min, and M. G. Emara. 2003. Mapping quantitative trait loci associated with resistance to coccidiosis and growth. Poult. Sci. 82:9–16.